AF/D

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Willem P. C. Stemmer *et al.*

Application No.: 09/437,726

Filed: November 9,1999

For: Methods for Obtaining a Polynucleotide Encoding a Polypeptide Having Rubisco Activity

Examiner: B. Sisson

Art Unit: 1634

Declaration of Genhai Zhu Pursuant to 37 C.F.R. 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Genhai Zhu, Ph.D., declare as follows:

1. I am currently employed as a Staff Scientist at Maxygen, Inc. I have been with Maxygen since 1998. At Maxygen I have worked as Project Leader in projects involving the genetic engineering of enzymes and metabolic pathways. I am currently Principal Investigator on a NIST-ATP grant project that aims to improve Rubisco and Rubisco activase by DNA shuffling.
2. In 1991 I received a Ph.D. in Plant Sciences from the University of Arizona at Tuscon, where my research was in the area of enzyme catalysis and kinetics and enzyme-ligand interactions.
3. From 1992-1994 I held a Research Associate position at the University of Nebraska-Lincoln, working in the laboratory of Robert J. Spreitzer (author of the Spreitzer reference discussed below), a recognized expert in the area of Rubisco and Rubisco engineering. My research in Dr. Spreitzer's laboratory focused on chloroplast transformation and the genetic engineering of Rubisco.

4. From 1994-1998 I held a Research Associate position at the University of Arizona , where I again conducted research in the area of chloroplast transformation and the genetic engineering of Rubisco.
5. I have authored numerous publications pertaining to the enzymology and genetic engineering of Rubisco. In particular, I have co-authored several publications with Dr. Spreitzer. A copy of my *curriculum vitae* is attached as Exhibit A.
6. I have reviewed the Office Action mailed July 16, 2002 in connection with the above-referenced patent application and the Minshull, Spreitzer, and Wolter references cited in the Office Action.
7. In my opinion, one of ordinary skill in the art at the time of invention would not have been motivated to apply the method of Minshull to obtain a polynucleotide encoding a Rubisco variant having enhanced Rubisco carboxylation activity. In November of 1998 (the priority date for this application) I had been actively involved in Rubisco research at a post-doctoral level for seven years. At that time, the general consensus in the field was that it would not be reasonable to expect success in screening a library of Rubisco variants for one with enhanced carboxylation activity. This view was based on the experience of those working in this area, who for many years had attempted to engineer and improve Rubisco. Their lack of success, and the very nature of the enzyme itself and the catalyzed reaction, strongly suggested that this approach would probably not be successful using the genetic engineering and screening technologies available at that time.
8. This state of affairs is reflected in the Spreitzer reference. In the Office Action, it is asserted that Spreitzer suggests finding "desirable clones," which I understand to mean Rubisco variants having enhanced carboxylation activity. However, when read in its entirety, Spreitzer actually teaches away from any attempt to screen a population of Rubisco variants for one having enhanced activity. In the section of Spreitzer cited by the Examiner (*Screening for Mutations*, page 416-18), the author is discussing screening for ***lethal*** Rubisco mutations, not Rubisco mutants having ***enhanced*** carboxylation activity as recited in the claims. The opening sentence of the *Screening for Mutations* section is "[e]ven though ***favorable Rubisco mutations cannot be selected*** directly, lethal Rubisco mutations would also be of value." The basis for Spreitzer's statement that mutants having enhanced carboxylation activity cannot be selected for directly can be found in the preceding section of

the paper entitled *Selection for a Better Enzyme*, pages 415-16. In that section, Spreitzer reviews a number of unsuccessful attempts that have been made to screen for improved Rubisco mutants. In an attempt by Somerville and Ogren, 5×10^6 plants were screened without finding a single one expressing an improved Rubisco. In a similar experiment, Spreitzer et al. screened greater than 1×10^9 mutagenized *C. reinhardtii* cells without finding a single Rubisco with improved catalytic constants. Pierce et al. are described as taking a more molecular approach, wherein their would be strong selection for a better enzyme under normal atmosphere – once again, no mutation was identified that improves Rubisco. Spreitzer concludes by stating that it will take more than a single amino acid substitution to make a better Rubisco, but if two specific amino acid substitutions are required simultaneously to make a better Rubisco, more than 1×10^{16} cells would need to be subjected to selection. It would be physically impossible to screen more than a tiny fraction of this number of cells.

9. As further support for my opinion, the Examiner's attention is directed to a review article entitled "Genetic Engineers Aim to Soup Up Crop Photosynthesis" (Mann, C.C., (1999) *Science* 283:314-16), attached as Exhibit B. Near the end of page 314 the author states that the quest for a better Rubisco is a Holy Grail in plant biology, but "[d]espite more than 20 years of effort, the hopes have not yet paid off." The author does find some basis for hope in recent advances in molecular biology and the unexpected discovery of a more efficient Rubisco in red algae. The author reviews each of these approaches, in each case pointing out the extreme technical difficulty and low predicted probability of success.
10. Significantly, the Mann article does not suggest or even mention using an approach that would involve screening for an improved Rubisco variant. This implies that the author viewed the likelihood of this approach succeeding to be even less probable than the approaches he does discuss, all of which he concedes are long-shots in terms of likelihood of success. This view is entirely consistent with the consensus opinion of those working in the field at the time, i.e., that an attempt to obtain an enhanced Rubisco by the screening of a library of variants would not be likely to succeed, and thus it would be worth trying alternate approaches that are technically challenging and not likely to succeed.
11. In another review article that published earlier this year (Gewolb, J. (2002) *Science* 295:258-59, attached as Exhibit C), the author states that "[d]ozens of research groups have [tried to

alter Rubisco] to improve the efficiency of photosynthesis, but none so far have succeeded." Thus, in spite of there being a long-felt need for an improved Rubisco, prior to the instant claimed invention the attempts have met with failure.

12. The Office Action asserts that Wolter et al. "disclose the shuffling of rubisco gene [sic] during evolution." Wolter et al. analyzed the sequences of known Rubisco genes and postulate that the structure of small subunit genes of $RbuP_2$ is the result of two counteracting processes working sequentially during evolution. First, introns were introduced before or during exon shuffling, adding new domains for new functions. Later, these introns were lost stepwise, leading to a more streamlined gene structure." (quoting the final paragraph). It is not the shuffling of exons that is noteworthy, but rather the introduction and stepwise loss of introns during the process. It is known in the field that "exon shuffling" is a general mechanism thought to occur during evolution, *i.e.*, it is not something specific or noteworthy with regard to Rubsico. Those working in the area of Rubisco enzymology would regard the teaching of Wolter as essentially irrelevant with respect to the question of whether it would be obvious to apply the teaching of Minshull to the problem of generating an improved Rubisco.
13. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the U.S. Code and that such willful false statements may jeopardize the validity of the patent application or any patent issuing thereon.

Dated: 10/28/02

Genhai Zhu, Ph.D.

Exhibit A

Curriculum Vitae

Genhai Zhu
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EDUCATION

Ph.D., 1991, Plant Sciences, University of Arizona, Tucson, Arizona.

EMPLYMENT

Staff Scientist, Maxygen, 1998 - present. Project leader, Genetic engineering: enzymes, metabolic pathways. PI, NIST-ATP grant project: Rubisco and Rubisco activase improvement

Research Associate, Department of Biochemistry, University of Arizona, 1994-1998. Genetic engineering of Rubisco enzyme, polyol metabolic pathways, multi-gene transformation, chloroplast transformation.

Research Associate, Department of Biochemistry, University of Nebraska-Lincoln, 1992-1994. Rubisco enzyme genetic engineering, chloroplast transformation.

Graduate Research Associate, Department of Biochemistry, University of Arizona, 1987-1991. Enzyme catalysis and kinetics, enzyme-ligand interactions.

GRANT AWARDED

1998, USDA/CSREES (New investigator award) for Rubisco research.

PUBLICATIONS (excluding abstracts)

Zhu G, Wildner GF, Schlitter J, Bohnert HJ, and Jensen RG (1998) Dependence of catalysis and CO_2/O_2 specificity of Rubisco on the carboxy-terminus of the large subunit at different temperatures. Photosynth. Res. 57, 71-79

Getzoff TP, Zhu G, Jensen RG, and Bohnert HJ (1998) Chimeric Arabidopsis thaliana L. Rubisco containing a Pisum sativum S is compromised in carbamylation. Plant Physiol. 116: 695-702

Zhu G, Wildner GF, Bohnert HJ, and Jensen RG (1998) Formation of the tight-binding inhibitor, 3-ketoarabinitol-1,5-bisphosphate by ribulose 1,5-bisphosphate carboxylase/ oxygenase is O_2-dependent. Photosynth. Res. 55, 67-74

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Flachmann R, Zhu G, Jensen RG, and Bohnert HJ (1997) Mutations in the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase increase the formation of misfire products. Plant Physiol. 114, 131-136

Zhu G, Jensen RG, and Bohnert HJ (1997) DNA sequence of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit from Arabidopsis thaliana (accession No. U91966) (PGR 97-074). Plant Physiol. 114, 395-396

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Spreitzer RJ, Thow G, and Zhu G (1995) Pseudoreversion substitution at large-subunit residue 54 influences CO_2/O_2 specificity of chloroplast ribulose-bisphosphate carboxylase/ oxygenase. Plant Physiol. 109, 681-685

Portis AR, Esau B, Larson EM, Zhu G, Chastain CJ, O'Brien CM, and Spreitzer RJ (1995) Characteristics of the interaction between Rubisco and Rubisco activase. In Photosynthesis: from light to bioshpere. Vol. V, ed. P. Mathis, Kluwer Academic Publishers, Dordrecht, pp 41-46

Zhu G, and Spreitzer RJ (1994) Directed mutagenesis of chloroplast ribulose 1,5-bisphosphate carboxylase/oxygenase: Substitutions at large-subunit Asn-123 and Ser-379 decrease CO_2/O_2 specificity. J. Biol. Chem. 269, 3952-3956.

Thow G, Zhu G, and Spreitzer RJ (1994) Complementing substitutions within loop regions 2 and 3 of the _/á-barrel active site influence the CO_2/O_2 specificity of chloroplast ribulose-1,5-bisphosphate carboxylase/ oxygenase. Biochemistry 33, 5109-5114.

Zhu G, Jensen RG, Hallick RB, and Wildner GF (1992) Simple determination of the CO_2/O_2 specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase by the specific radioactivity of [^{14}C]glycerate 3-phosphate. Plant Physiol. 98: 764-768.

Spreitzer RJ, Thow G, Zhu G, Chen Z, Gotor C, Zhang D, and Hong S (1992) Chloroplast and nuclear mutations that affect Rubisco structure and function in Chlamydomonas reinhardtii. In Research in Photosynthesis, Vol. III, ed. N. Murata. Kluwer Academic Publishers, Dordrecht, pp. 593-600.

Jensen RG, and Zhu G (1992) Rubisco fallover and negative cooperativity of substrate binding. In Research in Photosynthesis, Vol. III, ed. N. Murata. Kluwer Academic Publishers, Dordrecht, pp. 617-620

Zhu G, and Jensen RG (1991) Fallover of ribulose-1,5-bisphosphate carboxylase/oxygenase activity: Decarbamylation of catalytic sites depends on pH. Plant Physiol. 97: 1354-1358.

Zhu G, and Jensen RG (1991) Xylulose 1,5-bisphosphate synthesized by ribulose-1,5-bisphosphate carboxylase/oxygenase during catalysis binds to decarbamylated enzyme. Plant Physiol. 97: 1348-1353.

Zhu G, and Jensen RG (1990) Status of the substrate binding sites of ribulose bisphosphate carboxylase as determined with 2-c-carboxyarabinitol 1,5-bisphosphate. Plant Physiol. 93: 244-249.

EXHIBIT B

Among the more widely discussed biotech possibilities is altering the stomata, the porelike openings that stipple a plant's epidermis and control the in- and outtake of oxygen, carbon dioxide, and water. In most plants, the stomata are edged by two cells that resemble a pair of parentheses. When the plant takes in water, the stomatal cells swell open, allowing water to escape and permitting gas exchange; when the surroundings become drier or hotter, the stomata close. Because the stomata stay open longer than needed, most of the water that wheat and rice take in ends up in the atmosphere rather than being used in photosynthesis. "If you're irrigating, you might put up with the water loss in the name of getting the greatest biomass possible," says UC Davis's Loomis. "But if you're dry-land farming in Kansas, it might not be a good deal—you're using up water too fast."

To allow dry-land crops to use water more efficiently, stomata might be bioengineered to close more readily; in water-rich areas, they might be modified to stay open even longer. "That would give you better ventilation in the leaf, decreasing the canopy temperature and giving you better transport of CO_2, both of which could boost the rate of photosynthesis," says Fischer of the Australian Centre for International Agricultural Research.

Researchers have their eyes on two molecular targets that play a role in regulating the stomata: the plant hormone abscisic acid, which triggers closing, and an enzymatic process called farnesylation, which seems to impede ABA (*Science*, 9 October 1998, pp. 252, 287). By altering farnesylation, researchers may, in theory, be able to adjust plants' sensitivity to ABA and thus the tendency of the stomata to close. That task is daunting enough, but other researchers would like to go even further and tinker with the mechanisms of photosynthesis itself (see next story).

Many economists are confident that such efforts will eventually pay off and drive up crop yields again. But agronomists tend to view biotech as a long shot. Controlling such basic multigene traits, Fischer warns, is a "complex, unpredictable" task. Photosynthesis, notes Sinclair, is a process that evolution hasn't changed fundamentally "in a couple billion years." And even if the work is a technical success, the payoff may be minor, as traditional plant breeding has already pushed up crops' harvest index and ability to capture sunlight about as high as they can go. As Sinclair put it at the Irvine meeting, "Some of the hope for biotechnology seems analogous to the dreams of mechanical perpetual motion devices over a century ago: No matter how finely tuned the machine, reality does not allow output to exceed input."

Still, altering photosynthesis is "the great white hope" of the future of agriculture, as agricultural consultant Austin puts it. "All the relatively obvious steps have been taken. Photosynthesis is what's left."

Money woes

Re-engineering photosynthesis—or fundamentally improving crops in some other way—will require years of costly basic research, in Cassman's view. But a crucial source of support for agricultural science is eroding. For more than a century, according to Phil Pardey, an economist at IFPRI, government funding has supported long-term agricultural research. Although the biotech boom has spearheaded a recent massive increase in private-sector spending on agricultural R&D, notes Duvick, a former research director of agribusiness giant Pioneer Seeds, "even the big companies don't do a lot of long-term research."

But despite opposition from both the academic and corporate community, IRRI's budget in constant 1994 dollars has dropped from a high of $46.5 million in 1990 to $32.7 million in 1997, according to CGIAR figures. Similarly, CIMMYT's funding fell from $40.2 million in 1988 to $28.4 million in 1997. "We're taking away funding with the assumption that we've made it," says Dennis A. Ahlburg, a demographer at the London School of Hygiene and Tropical Medicine's Centre for Population Studies. "But if we don't continue to support [agricultural research], we'll slide backward."

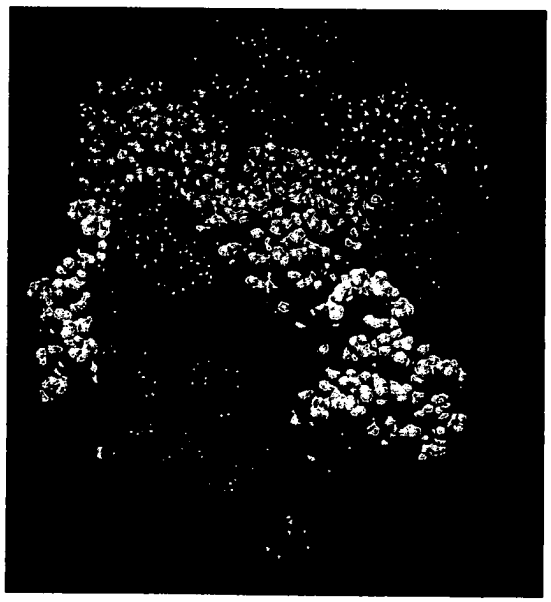
"The scientific challenge [of feeding the world] has been grossly understated," Cassman says. "But even if I'm wrong, and we somehow can do it without special effort, I think you'd like to have a margin of security. ... We are talking about the prospects for producing enough food to feed people in the next century, and a margin of security seems justified." —CHARLES C. MANN

FUTURE FOOD
▶BIOENGINEERING

Genetic Engineers Aim to Soup Up Crop Photosynthesis

To improve crops' ability to turn atmospheric carbon into food, researchers hope to alter the principal enzyme or supercharge it with CO_2.

Few nonbiologists may have heard of ribulose-1,5-bisphosphate carboxylase-oxygenase, the enzyme known as RuBisCO, but its importance is hard to overstate. The principal catalyst for photosynthesis, it is the basic means by which living creatures acquire the carbon necessary for life. By interacting with atmospheric carbon dioxide, RuBisCO—the world's most abundant protein—initiates the chain of biochemical reactions that creates the carbohydrates, proteins, and fats that sustain plants and other living things, ourselves included. But the enzyme also has another distinction, according to T. John Andrews, a plant physiologist at The Australian National University in Canberra: "RuBisCO is nearly the world's worst, most incompetent enzyme—it's almost certainly the most inefficient enzyme in primary metabolism that there is."



The enzyme that feeds the world. RuBisCO, which captures carbon dioxide and helps turn it into starches, sugars, and other compounds, is a target for genetic engineers.

CREDIT: *JOURNAL OF MOLECULAR BIOLOGY* 259, 160 (1996)

RuBisCO's ineffectiveness has been a spur to scientists since it became fully apparent in the 1970s. Indeed, the quest for a better RuBisCO is "a Holy Grail in plant biology," says George Lorimer, a biochemist at the University of Maryland, College Park, who worked with the Swedish team that mapped the enzyme's structure in 1984. "Everyone always goes in with the hope of changing the face of agriculture." Despite more than 20 years of effort, the hopes have not yet paid

off. But recent advances in molecular biology—and the unexpected discovery of more efficient RuBisCO in red algae—have given new impetus to the long struggle to modify the enzyme. In what may be the most ambitious genetic-engineering project ever tried, laboratories across the world are trying to improve the RuBisCO in food crops by either replacing the existing enzyme with the red algae form or bolting on what could be thought of as molecular superchargers.

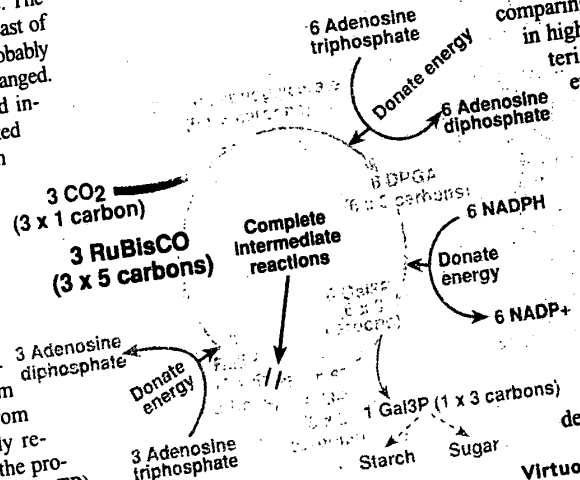
No one expects quick results—"I'm not for a second trying to minimize the task," says Andrews. The current state of the art in genetic engineering permits altering or splicing in single genes to improve a plant's resistance to pests, say, or to allow a crop to survive applications of weed-killing herbicide. To alter RuBisCO, by contrast, scientists must work with a 16-part molecule that is encoded by many genes in both the cell nucleus and the chloroplasts, where photosynthesis takes place. The enzyme also depends on a supporting cast of other enzymes, some of which will probably need to be revamped if RuBisCO is changed. But with many other avenues toward increasing crop yields seemingly blocked (see p. 310), RuBisCO has become an increasingly tempting target.

RuBisCO is just one actor in photosynthesis, which is a complex symphony of photochemical and enzymatic reactions. During the first, "light" stage of photosynthesis, chlorophyll—a green pigment in the chloroplasts—absorbs enough energy from sunlight to split off electrons from water molecules, simultaneously releasing oxygen gas and driving the production of adenosine triphosphate (ATP), which is used in the second, "dark" stage. This step begins when RuBisCO combines with carbon dioxide to produce 3-phosphoglycerate, or PGA (see diagram). Powered by energy from the ATP, a series of reactions transforms PGA into a host of starches, sugars, and other organic compounds.

Photosynthesis as a whole is not particularly efficient; a crop plant that stores as much as 1% of the total received solar energy is exceptional. As a result, the process offers many targets for bioengineers. But RuBisCO, far and away the biggest drag on the process, is the most appealing of them. First, it is torpid in the extreme—"perhaps the slowest known enzyme," William Ogren, a now-retired RuBisCO researcher from the University of Illinois, Urbana-Champaign, says with only slight exaggeration. Enzymatic rates are often on the order of 25,000 reactions per second; RuBisCO turnover in higher plants can be as little as two or three reactions per second. "Not one of evolution's finest efforts," says Ogren.

Second, RuBisCO triggers an additional reaction that interferes with the first. In 1971 Ogren and two other researchers discovered to their amazement that besides capturing and "fixing" carbon dioxide, RuBisCO catalyzes a second, opposing reaction. In what is called photorespiration, the enzyme combines with oxygen, rather than carbon dioxide, to create a compound that is subsequently converted partly into carbon dioxide. In other words, RuBisCO catalyzes one reaction that incorporates carbon into plants and another that ultimately strips them of carbon.

Typically, the RuBisCO in higher plants like rice and wheat is 100 times more likely to pick up CO_2 than O_2. But because the concentration of atmospheric O_2 is many times greater than that of CO_2, the greater affinity for CO_2 is largely canceled. As a result, 20% to 50% of the carbon fixed by photosynthesis is lost to photorespiration. "The oxygenation reaction is—as far as we can tell, and a lot of research over decades has gone into it—just a complete waste," says Andrews. "It doesn't do anything for the plant."



Virtuous cycle. The energy-carrying compounds ATP and NADPH, produced by photosynthesis, power a chain of reactions that begin when RuBisCO combines with carbon dioxide and ultimately produce starches and sugars.

This striking inefficiency was no handicap when photosynthesis first evolved 3 billion years ago, because the atmosphere was almost devoid of oxygen. After photosynthesis filled the air with oxygen and RuBisCO's weakness was revealed, it may have been too late for evolution to fix the problem, says Murray Badger, a RuBisCO specialist at The Australian National University. "It's a somewhat general correlation that the more specific and discriminatory a reaction becomes, the slower it gets," he says. As a result, mutations that made RuBisCO target carbon dioxide better might also have made it even slower.

If genetic engineers could find a way around RuBisCO's slowness and inefficiency, they might reap a double benefit. A faster, more efficient enzyme could help crops grow and increase their biomass, letting them produce more grain at a faster rate. In addition, explains Martin Parry of the Institute of Arable Crops Research-Rothamsted in Hertfordshire, Britain, RuBisCO's lethargy means that "plants need to invest incredibly heavily in it" to fix sufficient carbon. "A very large proportion of the plant's nitrogen requirements come from the need to produce the enzyme," which makes up as much as half the soluble protein in plant leaves. More efficient RuBisCO could thus lower crops' need for nitrogen, now mainly supplied by fertilizer in many countries.

A better RuBisCO. The discovery of photorespiration launched the effort to remodel RuBisCO. Researchers began by comparing the form of the enzyme found in higher plants with that in cyanobacteria—blue-green algae—which is even less efficient than the higher-plant version. To find out why, Lorimer's group, then based at DuPont, collaborated with Carl Branden's x-ray crystallography group in Sweden to determine the molecular structures of both forms, hoping to find telling differences. "We spent years creating high-resolution structures of spinach [a model plant in RuBisCO research] and cyanobacteria," says Lorimer. But despite the finely detailed results, "the sobering reality was that you can lay down [structural maps of] these two enzymes on top of each other and you're very hard pressed to see the difference." Even if the differences could be identified, Lorimer believes, they would be so numerous and subtle "that you could not rationally reason your way to what it was that you would need to improve the enzyme."

The failure of the structures to provide a path for modifying RuBisCO dismayed many researchers; Lorimer's group disbanded. Hopes reawakened in 1992, when F. Robert Tabita and B. R. Read of Ohio State University in Columbus discovered that some diatoms and red algae have more-specific RuBisCO than that in higher plants. In 1997, a team led by Akiho Yokota, a plant molecular physiologist at the Research Institute for In-

—CHARLES C. MANN

With reporting by Dennis Normile in Tokyo.

SOURCE: HELENA CURTIS, BIOLOGY (WORTH PUBLISHERS, 1983)

novative Technology for the Earth, in the Keihanna Science City near Osaka, Japan, found red algae with RuBisCO that is about three times more efficient. "We've looked at a lot of the red algae," Tabita says, "and the trend is always the same, 2 1/2 to threefold higher than normal plants." No one yet knows why.

To try to exploit this advantage, Andrews's group is one of several that are attempting to insert RuBisCO genes from red-algal chloroplasts into the chloroplasts of higher plants, using techniques for manipulating chloroplastic DNA developed by Rutgers University biochemist Pal Maliga. "If it can be done, it would be really amazing," says Yokota, who also works at the Nara Institute of Science and Technology, in Kansai Science City, near Nara, Japan. Other groups are working on related approaches at Rothamsted, Ohio State, and the University of Nebraska, Lincoln.

"This is a little bit like transferring a V-8 engine from a big automobile into a small car," says Andrews. "It may not work." Even if the enzyme functions in its new, transgenic home, he cautions, "it's not enough simply to get the RuBisCO in there; it has to be assembled and produced in the right form, and also be connected to the regulation system that the chloroplast keeps control of RuBisCO with." Andrews hopes to see results in "about 10 years."

Fired-up photosynthesis. Red algae like this one have a RuBisCO that is as much as three times more efficient than the enzyme in green plants.

Supercharging photosynthesis. While most researchers trying to modify the genetic basis of photosynthesis are focusing on RuBisCO, a few are trying another, perhaps even more ambitious, strategy. Just as small engines can go faster if they are equipped with a supercharger, which force-feeds them with fuel, some plants have their own photosynthetic supercharger, known technically as the C_4 cycle. In C_4 plants, the bundle cells where photosynthesis takes place are surrounded by specialized "mesophyll" cells, which temporarily fix carbon dioxide and jam it into the bundle cells at such high concentrations that the oxygen reaction is effectively blocked. The C_4 cycle requires so much energy that C_4 plants cannot grow in dim light, but in the right, well-illuminated conditions, C_4 crops like sugarcane photosynthesize more efficiently than any others. About 5% of all terrestrial higher-plant species use the C_4 cycle; maize is economically the most important.

A joint team at Japan's National Institute of Agrobiological Resources and Nagoya University led by Nagoya microbiologist Makoto Matsuoka is now attempting to reproduce the C_4 cycle in rice. For the transformation to succeed, a host of altered enzymes would have to work together properly, and the plant's structure may have to be changed to create the equivalent of mesophyll cells. As a result, the project may well be the most fundamental genetic alteration that humankind has ever tried in any organism. "Don't hold your breath," Lorimer says.

Indeed, Matsuoka cautions, "I don't think we can really make a true C_4 [rice] plant." Rather than transferring the whole genetic structure for the C_4 cycle from, say, maize into rice, his team is trying to identify nonfunctioning equivalents of C_4-type genes in rice and selectively replace them with their active counterparts from maize.

In a paper in press at *Nature Biotechnology*, Matsuoka's group reports taking a first step by replacing three silent rice genes with their more lively equivalents in maize, including the important enzyme phosphoenolpyruvate carboxylase (PEPC), which catalyzes the beginning of the C_4 cycle. "We succeeded in getting [PEPC] highly expressed in a rice plant," Matsuoka says. "This is a world first." After transferring each gene to a different rice plant, the group is now crossing the results to obtain rice that produces all three enzymes.

That may not be enough to replicate C_4-like photosynthesis in rice, says Matsuoka. Rice has mesophyll-like cells that are not photosynthetically active, and these may have to be activated. And some of the changes may actually be deleterious—the transgenic rice with only the C_4 enzyme NADP-malic tends to die quickly, for example. Still, Matsuoka says, preliminary evidence suggests that active PEPC in rice cuts the destructive oxygen reaction by about a third.

Even as the work to alter photosynthesis begins to gain momentum, some critics question whether it will benefit agriculture. Since at least 1970, research has shown little correlation between crops' photosynthesis rates and their yields, suggesting that improvements in RuBisCO won't automatically translate into better harvests. But according to Steven P. Long, a plant physiologist at the University of Essex in the U.K., the correlation may simply be hidden by the propensity of higher yielding cultivars to have bigger leaves, which increases the amount of self-shading and thus lowers the mean photosynthetic rate. When he and his colleagues temporarily boosted photosynthesis rates in wheat by flooding open fields with enough CO_2 to increase local atmospheric levels by 50%, grain yield went up 10% to 12% in two consecutive growing seasons.

Long is more skeptical about the value of importing the C_4 cycle into crops like wheat and rice. Because the C_4 cycle imposes a high energy cost on the plant's metabolism, it only pays off at higher temperatures—that's why there is no winter maize crop. "You can model it fairly easily," Long says. "Below 28°C, the [standard photosynthesis] is more effective, and above 28°C the C_4 is more efficient." The payoff threshold will rise even higher as the atmosphere's CO_2 concentration increases because of human activity. And if scientists like Andrews succeed in increasing the specificity of RuBisCO, the threshold for adding the C_4 cycle will go still higher—perhaps to 40°C, he says. "There'd be no point in going to C_4 then."

Nonetheless, Long favors working on both approaches. "These are such major steps that we don't even know how many unknowns there are in doing this. Pursuing all options is well worthwhile."

"We've now reached the limits of what we can do [with conventional breeding]," Rothamsted's Parry says. "So therefore we have to solve the next problem, which is putting in a bigger engine." It's a challenge, he observes, "with considerable practical interest." —CHARLES C. MANN

With reporting by Dennis Normile in Tokyo.

CREDIT: PHOTOTAKE/PNI

EXHIBIT C

studies have produced unexpected results. Only a decade ago, for example, many geologists assumed that as the climate grows warmer and wetter, erosion and chemical weathering (the chemical breakdown of soil and rocks) accelerate. But this notion has itself been slowly eroding. Clifford Riebe, a geomorphologist at UC Berkeley, has used cosmogenics to produce some of the first quantitative evidence that challenges the old assumption. When he compared weathering rates with long-term erosion rates at seven sites that span wide climate ranges in California's Sierra Nevada mountains, Riebe found that erosion and weathering did indeed go hand in hand but that climate had little effect on either. "Given that chemical weathering gets its name from weather," Riebe says, his results "came as a surprise."

Instead, Riebe discovered, erosion was swiftest on steep slopes near geologic faults or river canyons—evidence that tectonic activity eclipsed climate in driving erosion and weathering. Extrapolated to a global scale, that conclusion bolsters a 13-year-old theory that the uplift and erosion of the Himalayas—and the ensuing consumption of atmospheric CO_2 by chemical weathering reactions—triggered a global cooling that began about 40 million years ago. Riebe is now roaming from the rainforests of New Zealand to the deserts of Mexico to see whether tectonics dominates weathering rates even in those extreme climates.

As cosmogenic studies of erosion move out of the hands of specialists and into more widespread usage, new applications are blossoming. Researchers like Vermont's Bierman readily rattle off long lists of their plans and predictions for future research. "Overall, what keeps this exciting for me is [applying the techniques to] problems that we otherwise haven't been able to solve," Bierman says. "It's not an incremental learning experience. It's a major learning experience."

—LIESE GREENSFELDER

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BIOENGINEERING

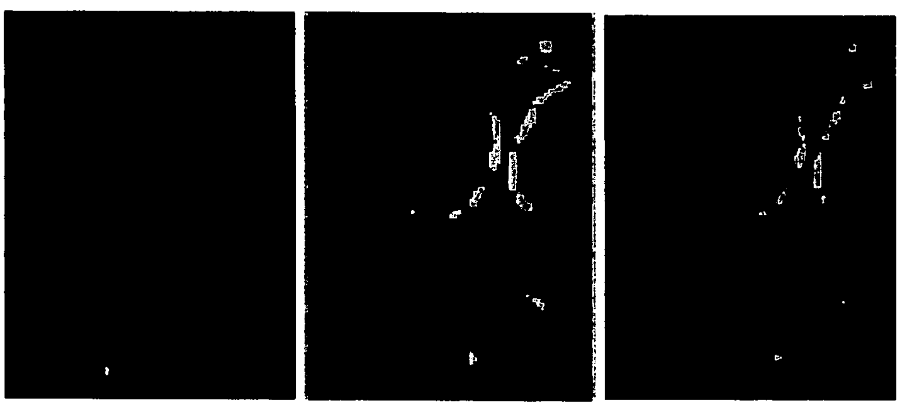
Plant Scientists See Big Potential in Tiny Plastids

Tinkering with plant cells' second genome could boost photosynthesis or turn plants into drug factories

When it comes to genetic engineering, the genes inside the nucleus get all the attention. But plants have an unassuming second genome inside tiny organelles called plastids. And although this small, circular genome carries far fewer genes than its nuclear counterpart, researchers say its potential for genetic engineering far outstrips its size.

The plastid genome arose some 1.5 billion years ago, when the ancient ancestors of modern plants are thought to have engulfed photosynthetic bacteria and put them to work manufacturing food. Over time, many of the original plastid genes slipped into the nucleus, but a small genome with about 100 genes remains. Plants now contain undifferentiated organelles that can diversify into a number of specialized plastids, each of which carries this secondary genome. The most famous member of the plastid family is the chloroplast, the photosynthesis factory. Others include the amyloplasts, which store starch, and the oil-producing elaioplasts.

Because plastids present an easy way to produce proteins in high concentrations and offer unique access to the photosynthetic machinery, some plant scientists believe they offer some of the best opportunities to make transgenic crops that grow more efficiently, for instance, or that manufacture medicines. "You get high yields" of proteins produced by plastids, says emeritus Harvard molecular biologist Lawrence Bogorad. And when it comes to boosting photosynthesis, "you can probably do things in the chloroplast compartment that you can't do in the nuclear compartment," Bogorad says. But transforming plastids is technically tricky, and the field, although growing, remains small.



Neon plastids. When inserted into the plastid genome, a gene for a fluorescent marker protein (GFP) signals a successful transformation.

The promise of plastids

For years, researchers have dreamed of tinkering with the genes in plants to turn them into living, photosynthesizing drug factories. If plants could be engineered to pump out lots of therapeutic proteins, these could be isolated and made into medicines. But, although creating transgenic plants by altering their nuclear DNA has become routine, it remains extremely difficult to get these plants to produce the desired protein—say, antibodies against herpesviruses or enzymes for diagnostic kits—in large quantities. In most such plants, the new protein accounts for a paltry 1% of the plant's total protein output, although levels as high as 25% have been reported in a few exceptional cases.

Transgenic plants made with altered plastids are much more productive than nuclear-engineered plants. Last year, geneticist Henry Daniell of the University of Central Florida in Orlando inserted a gene cluster for an insecticidal *Bacillus thuringiensis* toxin into the chloroplasts of tobacco plants; the chloroplasts churned out vast amounts of the crystallized protein—45% of the cell's total protein output. Levels routinely reach 5% to 15% in the latest studies, says geneticist Pal Maliga of Rutgers University, New Brunswick, New Jersey.

Engineering the plastid genome has additional advantages over nuclear transformation. For example, the risk that foreign genes introduced into plastids will spread to other plants is much lower than the risk that nuclear genes will make such a leap. This is because plastid DNA in most crop species is transmitted only from generation to generation through the ovules, the plant "egg," not through pollen, the plant "sperm"—just as animals' mitochondrial DNA is passed down only through the egg. Plants produce

thousands of tiny pollen grains that can be spread uncontrollably by wind or insects over wide distances, but seeds developing from ovules stay with the plant.

What's more, the rules of molecular biology are different in the plastid than in the nucleus. In the nuclear genome, each gene is turned on and off by its own control sequence. That makes it difficult to engineer complex traits controlled by many genes. But in the plastid, multiple genes are controlled by the same genetic switch, as is the case in bacteria. "It's like a bacterial fermenter in a plant cell," says research director Peter Heifetz of the Torrey Mesa Research Institute in San Diego. With plastids, he sums up, "you solve a lot of problems in one shot."

Technical difficulties

But plastid genomes have often defied the best efforts to modify them. Since Maliga modified the plastid genome of tobacco more than a decade ago, scientists have been able to transform plastid genomes in just a handful of plant species. The technical knowledge required to rework plastids is spreading, but slowly.

Modifying the plastid genome is tough for the same reason that it's promising: Tens of thousands of copies of the genome may be present in any given cell. A single plastid can have hundreds of copies of the genome, and a plant cell can have hundreds of plastids. For successful transformation, the new gene must be present in each copy of the plastid genome within each cell.

To achieve that, scientists first insert their gene of choice into a single plastid and then allow the cell to divide many times in culture. Then they apply a delicate balance of chemicals that allows cells with more copies of the gene to prosper. After months of selection—if all goes well—the culture will contain only transformed cells.

At this point, scientists are left with a plate of undifferentiated cells that they have to turn back into a plant. Doused with the proper cocktail of plant hormones, tobacco, potato, tomato, and other plants in the nightshade family are easy to regenerate—and plastid engineering has been relatively successful in them. But many of these techniques are species-specific, and it's been difficult to apply them to other plants.

Healthful tobacco?

So far, tobacco has yielded the most plastid engineering successes. In 2000, plant geneticist Jeffrey Staub and his colleagues at Monsanto in St. Louis genetically altered tobacco chloroplasts so that they produced a correctly folded human protein called somatotropin, which is used to treat dwarfism in children. Maliga calls the findings a "milestone," because they convinced skeptics that the bacteriumlike genetic machinery in plastids was capable of correctly folding mammalian proteins. The study also showed that the plastid-engineered plants don't modify proteins after synthesizing them—a major drawback when making human proteins in a plant's nucleus. Monsanto does not plan to commercialize the plants, which produce the protein at levels 300-fold higher than do their nuclear transgenic counterparts. The researchers chose to work with somatotropin, which has a well-studied structure, only as proof of principle; however, the company is now rumored to be working on expressing other human proteins in tobacco. "We may actually find something very useful to do with tobacco," says retired Duke geneticist Nicholas Gillham.

Shooting up. After researchers weed out nontransformed tomato cells, they sprout the cells with souped-up plastids.

Tobacco isn't an ideal host, however. For one, it grows in a restricted geographical area. But, more important, it would be difficult to extract proteins of interest from the plants, which produce other troublesome compounds such as nicotine.

Making a fruit or tuber with genetically transformed plastids would get around many of tobacco's limitations. The edible result would be big enough to contain large quantities of the compounds of interest. In 1999, Staub and his Monsanto colleagues produced potato plants with genetically modified plastids. But the tubers expressed the foreign genes at a concentration 100 times lower than that in the leaves, equivalent to levels achievable by nuclear transformation.

Engineered tomatoes have met with more success. Ralph Bock and his colleagues at the University of Freiburg, Germany, spent more than 2 years developing transformation and regeneration conditions for a tomato. In the September 2001 issue of *Nature Biotechnology* they report proof of principle that the tomato fruit can be modified. The 1% expression levels the researchers achieved were fairly low, but on the bright side, the fruits produced fully half as much protein as the plants' green leaves. This suggests that the same tricks that help increase expression levels in tobacco leaves might lead to fruits that express engineered plastid proteins in large quantities. "These are first-generation expression levels," says Heifetz, who expects that the researchers will be able to improve the fruits' output.

Future harvests

Photosynthesis is very inefficient, turning less than 1% of the incoming solar radiation into food. If it could be slightly improved, the face of agriculture would change dramatically, as plants could grow bigger with less sunlight.

Until the invention of plastid transformation, scientists could not tinker with RuBisCO, the key carbon-fixing enzyme of photosynthesis, because half of its subunits are encoded in the chloroplast genome. Dozens of research groups have since tried to alter the molecule to improve the efficiency of photosynthesis, but none so far have succeeded (*Science*, 15 January 1999, p. 314).

Now, however, plant physiologists Spencer Whitney and T. John Andrews at Australian National University in Canberra have taken a key step toward this goal, creating the first viable plants with an altered RuBisCO. They report in the 4 December 2001 issue of the *Proceedings of the National Academy of Sciences* that photosynthetic efficiency drops predictably when the RuBisCO of tobacco plants is replaced with the less efficient RuBisCO from the red alga *Rhodospirillum rubrum*. Even though the plants are less efficient, not more, the advance is exciting, according to Maliga, because it is "the first time that [researchers have] actually changed the properties of the photosynthetic machinery in a predicted fashion."

Andrews ties the success of the study to the spread of the technical skills needed to conduct plastid transformation. "I think it hasn't been done before because the technology for plastid transformation is not that widely dispersed," says Andrews. As that knowledge becomes more widespread, the humble plastid may acquire mightier powers.

—JOSH GEWOLB

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